REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
30-08-2013	Final Report	1-Jun-2010 - 31-May-2013

4. TITLE AND SUBTITLE
Development of Synthetic Spider Silk Fibers for High Performance Applications

5a. CONTRACT NUMBER
W911NF-10-1-0169

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
Breslauer DN

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Refactored Materials, Inc.
344a Prentiss St
San Francisco, CA 94110 -6141

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
57821-LS.3

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
The overall goal of this project is to demonstrate the feasibility of synthetic production of high-performance spider silk fibers for use in next-generation automotives, aerospace, body and vehicle armor, ropes, webbing, and advanced composites. This requires creating a system for producing the protein-based material with sufficient efficiency that, scaled up, would be commercially viable. Using cutting edge techniques in synthetic biology (e.g. gene design and synthesis), we created multiple type and multiple size silk sequences for maximum expression in a

15. SUBJECT TERMS
silk, synthetic biology, fiber, protein materials

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Daniel Widmaier
UU	UU	UU	UU		19b. TELEPHONE NUMBER 415-279-5585

Report Title

Development of Synthetic Spider Silk Fibers for High Performance Applications

ABSTRACT

The overall goal of this project is to demonstrate the feasibility of synthetic production of high-performance spider silk fibers for use in next-generation automotives, aerospace, body and vehicle armor, ropes, webbing, and advanced composites. This requires creating a system for producing the protein-based material with sufficient efficiency that, scaled up, would be commercially viable. Using cutting edge techniques in synthetic biology (e.g. gene design and synthesis), we created multiple type and multiple size silk sequences for maximum expression in a scalable, high expression recombinant organism. We created laboratory scale quantities of silk protein feedstock to determine the feasibility of synthetic spider silk production for commercial uses.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
08/31/2012	1.00	David N. Breslauer, David L. Kaplan. Silks: Properties and uses of natural and designed variants, Biopolymers, (06 2012): 0. doi: 10.1002/bip.22007
TOTAL:	**1**	

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received		Paper
TOTAL:		

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Paper

TOTAL:

Patents Submitted

U.S. Patent Application No. 61/716,890, “Cellular Reprogramming for Product Optimization.” Daniel Widmaier and David Breslauer.

Under filing, “Methods and Compositions for Synthesizing Improved Silk Fibers.” Daniel Widmaier, David Breslauer, and Joshua Kittleson.

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
David Breslauer	0.20
Daniel Widmaier	0.46
Ethan Mirsky	0.11
Joshua Kittleson	0.08
FTE Equivalent:	**0.85**
Total Number:	**4**

Sub Contractors (DD882)

Inventions (DD882)

5 **Cellular Reprogramming for Product Optimization**

Patent Filed in US? (5d-1) N

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2):

5a: David Breslauer

5f-1a: Refactored Materials

5f-c: 409 Illinois St.

San Francisco CA 94158

5a: Daniel Widmaier

5f-1a: Refactored Materials

5f-c: 409 Illinois St.

San Francisco CA 94158

5 **Methods and Compositions for Synthesizing Improved Silk Fibers**

Patent Filed in US? (5d-1) N

Patent Filed in Foreign Countries? (5d-2) N

Was the assignment forwarded to the contracting officer? (5e) N

Foreign Countries of application (5g-2):

5a: David Breslauer

5f-1a: Refactored Materials

5f-c: 409 Illinois St.

San Francisco CA 94158

5a: Joshua Kittleson

5f-1a: Refactored Materials

5f-c: 409 Illinois St.

San Francisco CA 94158

5a: Daniel Widmaier

5f-1a: Refactored Materials

5f-c: 409 Illinois St.

San Francisco CA 94158

Scientific Progress

See Attachment

Technology Transfer

Development of Synthetic Spider Silk Fibers for High Performance Applications

Refactored Materials, Inc.

Grant W911-NF-10-1-0169

Final Report

June 1, 2010 – August 31, 2013

Table of Contents

Statement of Problem Studied

The overall goal of this project is to demonstrate the feasibility of synthetic production of high-performance spider silk fibers for use in next-generation automotives, aerospace, body and vehicle armor, ropes, webbing, and advanced composites. This requires creating a system for producing the protein-based material with sufficient efficiency that, scaled up, would be commercially viable. Using cutting edge techniques in synthetic biology (*e.g.* gene design and synthesis), we created multiple type and multiple size silk sequences for maximum expression in a scalable, high expression recombinant organism. We created laboratory scale quantities of silk protein feedstock to determine the feasibility of synthetic spider silk production for commercial uses.

Summary of Most Important Results

Year 1

Summary

- Developed novel, proprietary software algorithms and assembly methods to optimize and construct highly repetitive GC-rich DNA for introduction in a recombinant host.
- Designed and assembled four differently encoded variants of MaSp1 gene fragments into 1/4 length silk genes, complete with N- and C-termini.
- Transformed all protein variants into a proprietary yeast strain and screened for expression. While all encoded variants expressed to some degree, a clear preference was seen for certain encodings demonstrating that gene coding matters.
- Spun initial fibers from our proteins.

Gene Design of a Major Amputate Spidroin (MaSp1) Protein

Silk proteins are extreme outliers when compared with normal cellular protein, they are highly repetitive and comprised of only a limited set of amino acids. It was unclear whether traditional sequence-optimization algorithms, such as the commercial gene synthesis supplier, DNA20's, would be effective on silks. Therefore we tried several different optimization approaches. The four variants are identical in amino acid sequence, but differ in codon selection. The variants include a wild-type sequence for control purposes, one sequence generated by a commercial algorithm (DNA20) and two generated in-house by Refactored Materials.

For each codon selection, an 8-repeat block segment (one-twelfth full-length) section was designed, with the N- and C-terminal non-repetitive regions added. This length was chosen because it captures vast majority of sequence variation of the natural protein and can therefore be used as a building block without loss of any critical protein structure. In comparison, previous approaches to silk gene design relied on 1-repeat block regions that do not fully capture the natural

protein structure[1-8]. In addition, this length was chosen as the best compromise between the desire for larger block segments and the limitations of commercial gene synthesis, for which long, highly repetitive genes are generally prohibitively difficult and expensive (>$3/base). Each 8-repeat block segment was then synthesized by commercial gene synthesis, in a form ready to assemble into larger silk sequences.

Gene Assembly

Most methods for the large-scale assembly of DNA have difficulty with long, repetitive, and GC-rich DNA sequences and introduce extraneous basepairs ("scars") between segments, limiting the broad range applicability of these techniques, particularly in modularly assembling precision protein polymers[9]. In particular, scars inhibit the ability to faithfully replicate native sequences that are critical for reconstituting bioactivity and mechanical properties (Figure 1a).

To resolve this problem, we developed a gene assembly method capable of scarlessly assembling the long-length, repetitive, and GC-rich DNA that is inherent in structural proteins. The assembly method involves sequentially adding each 800 bp segment and modifying the ends of the DNA to allow for seamless addition of the next block. This method has a relatively long turn-around time, but enables the assembly of arbitrarily long sequences, as well as the insertion of any desired combination of components.

All four variants of the 8-block sequences designed and synthesized as described above were assembled into 24-block (quarter length) silk genes, complete with N- and C-termini (Figure 1c).

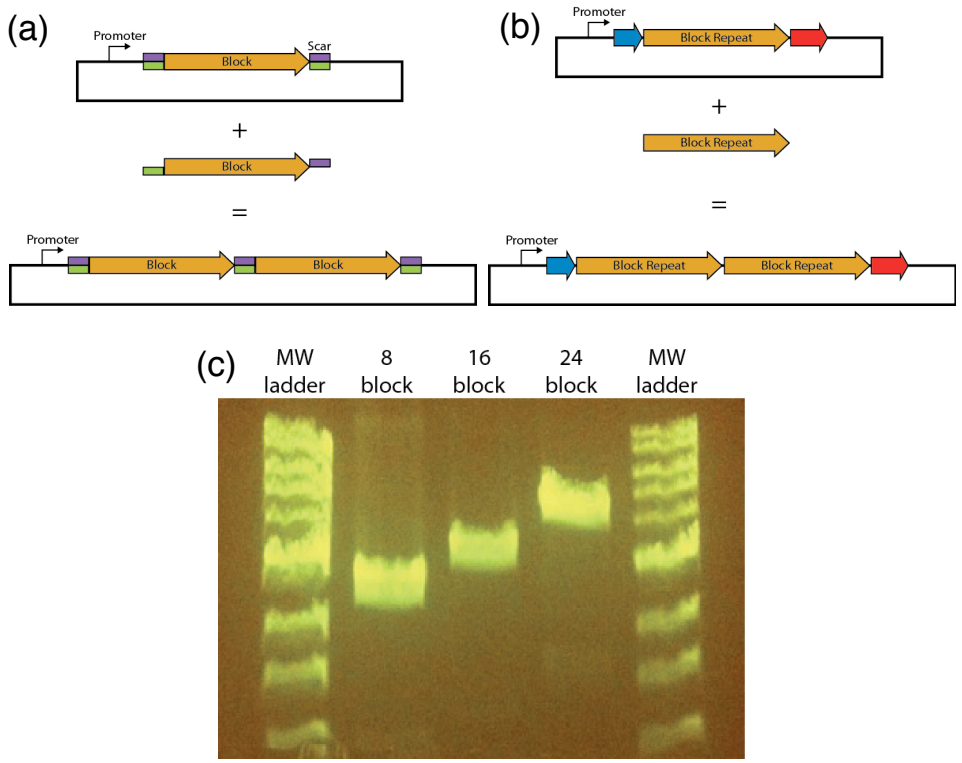

Figure 1. Initial Refactored Materials' DNA Assembly Method. (a) Diagram of standard DNA assembly techniques that use overhangs to assembly blocks, leaving scars in the final assembly. (b) Diagram of repetitive gene assembly method. Blocks can be inserted scarlessly into a vector. (c) Demonstration of increasing length spider silk genes constructed using the assembly technique.

Organism Selection, Optimization and Screening Method

Previous efforts to create recombinant silk have used a wide variety of expression organisms, ranging from bacteria to plants to insects to mammals[1-6,10-12]. Among the most successfully expressing organisms has been the methylotropic yeast *Pichia pastoris.* Yeast are an attractive organism for recombinant protein expression because they are eukaryotes capable of efficiently producing large proteins, they perform post translational modifications, recombinant proteins can be secreted into their culture media, and they are well adapted to high density fermentation. In addition, *P. pastoris* is capable of growing at high density on low-cost mineral media, a critical requirement for creating a scalable and commercially viable production method. Genes can be integrated into the genome under the control of the methanol sensitive AOX1 promoter. This is a well-recognized industrial system for the heterologous expression of recombinant proteins at high yield[3,13-15].

To further enhance our ability to produce large quantity of silk, we created a custom-engineered strain of *Pichia* that is optimized for expression of silk proteins. Modifications include an enhanced ability to select correctly inserted

(transformed) recombinant DNA in the yeast genome and a metabolic alteration to enhance protein expression on methanol media.

Unlike bacteria, *Pichia* transformants do not each express protein at the same amounts. Due to metabolic variations, different colonies have different expression levels. Therefore to find a high-expression strain, or to compare different DNA sequences for expression level, several colonies must be screened for the protein of interest. We developed a method for screening silk proteins utilizing Western and dot-blots.

Protein Production Method Evaluation

All four 24-block constructs described above were transformed into this yeast strain and dozens of colonies screened for expression of MaSp1. A sample of the results is shown in Figure 2. Overall, there was a significant variation in expression levels between codon optimization methods, with the wild-type sequence expressing the worst, and codon usage pattern matching being the best.

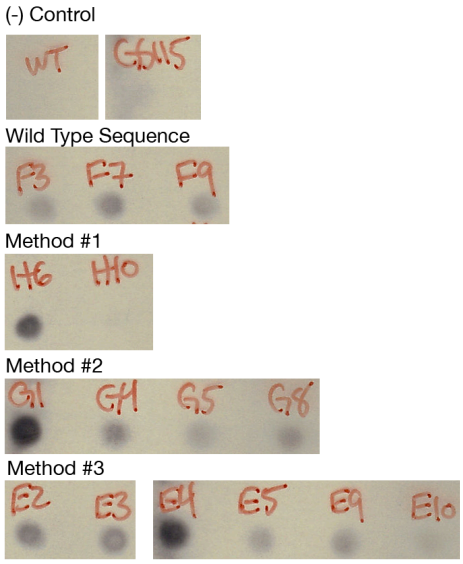

Figure 2. Effects of DNA encoding on protein expression. Each panel shows cellular lysate from the indicated clone probed for the presence of silk protein. A darker spot corresponds to the presence of more silk protein. The negative control strains show no protein while the wild-type sequence shows consistently low amounts of MaSp silk protein. Codon optimization methods #1-3 are different ways of selecting codons in an attempt to improve expression level. Each method shows improvements of expression over wild-type with much higher possible silk protein levels, however these methods also appear to increase clone-to-clone variability. These are representative results for multiple silk proteins tested with these codon optimizations.

In addition to identifying the optimal DNA sequence, the best producing strains were selected and grown in liter-scale flasks to create protein for fiber spinning experiments. The initial silk constructs were created with a 6xHIS tag (six histadines) to bind nickel/cobalt affinity purification columns. The yeast cells were lysed and the silk separated by affinity purification from other cytosolic protein. This method was selected for its reliability and utility for experimentation.

Initial protein yields were estimated at ~100 mg/L per multi-day culture in low-density shake flasks. This was a major improvement over our titers of ~1-10 mg/L in the academic lab prior to starting this project. 10mg of silk protein was purified, dissolved to ~10% w/v in hexafluoro-2-propanol, and extruded into a 100% methanol coagulation bath (Figure 3).

Figure 3. Initial fiber spun from 10mg of intracellularly expressed silk protein. Fiber is approximately 1mm in diameter.

Year 2

Summary

- Re-engineered constructs for extracellular secretion.
- Tested multiple length silks for secretion.
- Determined production-limiting elements of silk N-terminal domain.
- Determined process conditions to produce silk protein at fermenter scale.
- Produced sufficient quantities of silk protein to attempt to make materials.
- Began small-scale fiber spinning experiments.

Strain and Construct Improvement

We further enhanced our *Pichia* strains and constructs for the secretion of silk protein, outside of the cell, rather than for intracellular expression. This is accomplished by fusing the *Saccharomyces cerevisiae α*-mating factor (*α*Mat) to the N-terminus of the protein. This tag is natively cleaved off as the protein travels through the secretion pathway, and is well-established as a secretion tag in *Pichia*[15].

The secretion of protein into the media vastly improves the ease and economics of protein production. There is no need for cellular disruption to recover the protein, and *Pichia* secretes only one major protein, that can be non-

detrimentally knocked out[16]. As such, secreted protein in the media is highly pure relative to intracellular production.

Initially, to detect and purify our silk protein, we had fused 3xFLAG and 6xHIS affinity tags to the C-terminus of our proteins. This is standard practice in molecular biology, and has also been commonly performed with recombinant silks[11]. However it is believed that a 6xHIS tag, because of its strong positive charge, could be detrimental to fiber formation and properties. As such, we re-engineered our silk constructs and detection methodologies such that we removed the 6xHIS tag, leaving only the 3xFLAG tag.

High-throughput Screening for Protein Production

Unlike bacteria, *Pichia* transformants do not each express protein at the same amounts. Due to metabolic variations, different colonies have different expression levels. Therefore to find a high-expression strain, or to compare different DNA sequences for expression level, several colonies must be screened for the protein of interest. As discussed, we intially developed a method for screening silk proteins utilizing Western and dot-blots, from *Pichia* transformants grown in shake flasks. The method was capable of screening tens of colonies at a time. We have since developed a partially automated process-- using a colony picker, liquid handling robot, and plate shaker/incubator-- for the growth, induction, and analysis in 96-well plate format (Figure 4). We can currently screen ~20,000 colonies/week. In addition to the ability to screen many different silk constructs (as discussed in depth below), we know from literature,[17,18] private communications, and our own preliminary research that if a sufficient number of colonies are screened, transformants can be found that secrete a titer ~10x higher than the average of all of the colonies. We utilize our extra screening capacity to seek out high secreting clones to improve the economics of our silk production.

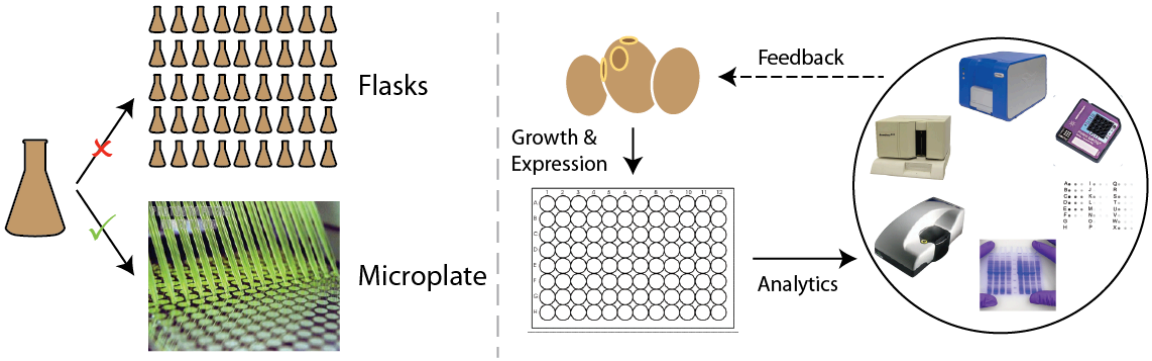

Figure 4. (left) Screening multiple clones in flasks is time and cost prohibitive. Microplate formats have been used successfully in the past to screen tens of thousands of colonies for high expressions. (right) The samples from the plate assay are fed into various analytics platforms. This data guides the reengineering of strains and subsequent high-throughput experiments.

Combinatorial Assembly of Sequence Components

Silk proteins can be divided into three component domains: the N-terminal domain (NTD), the block repeat region, and the C-terminal domain (CTD) (Figure 2, top). The NTD and CTD are well-studied and are believed to confer aqueous stability, pH sensitivity, and molecular alignment upon aggregation[19]. NTD also has a strongly predicted secretion tag, which is often removed during heterologous expression[20]. The repetitive region composes ~90% of the protein, and folds into the crystalline and amorphous regions believed to confer strength and flexibility to the silk fiber, respectively[8].

We set out to determine which of these components, and which physical properties (such as molecular weight), contribute to the expression, secretion, stability, and aggregation of silk constructs transformed to secrete from *Pichia*. We developed a combinatorial library of silk constructs based on these components. These building blocks consisted of NTD, CTD, and an 8-repeat block segment (one-twelfth full-length) section of the silk protein. To create longer proteins, we concatenated 8-block (8B) repeat sequences. For example, constructs we have assembled, transformed and tested for secretion include NTD, 8B, 8B-CTD, NTD-8B, 24B, 24B-CTD, and 60B-CTD.

These experiments generated an abundance of data. The broader conclusions, in regards to silk production, that have resulted from this work are:

- NTD has strong effects on expression, processing, and secretion
- CTD alters stability and aggregation behavior, compared to constructs of repetitive blocks alone
- Longer repeats (>24B) are highly susceptible to aggregation and proteolytic degradation
- *Pichia* can readily secrete >208 kDa heterologous proteins (currently our largest construct tested). Native *Latrodectus* MaSp1 is ~320 kDa and the largest reported recombinant silk is ~285 kDa[21].

Multi-liter Scale Silk Production and Silk Processing

We began process development of out silk secreting strains in our fermentation vessels. The sensors and feedback control in the fermenters allow us to tightly regulate the cell growth and secretion environment. From these experiments we have discovered that the addition of surfactant, and regulation of pH is critical for maintaining silk in a non-aggregated, soluble state (Figure 5). The specific pH to prevent aggregation is construct-dependent, largely dependent on whether or not the construct has the C-terminal domain. We furthermore improved fermentation conditions to minimize proteolytic degradation, enabling production of gram-scale quantities of longer silk constructs.

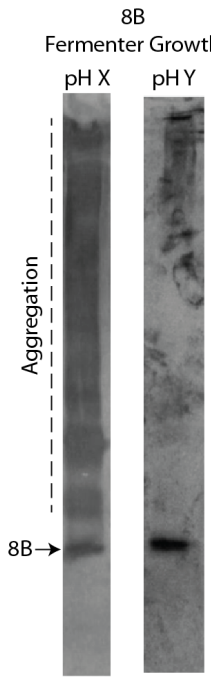

Figure 5. Western blots of 8B grown in fermenters under two different tightly regulated pH levels. Aggregation (higher molecular weight smearing in the lane) is highly pH dependent.

We produced hundreds of milligrams of 8B (21 kDa). We found that, regardless of buffer conditions, the protein was not spinnable, even if left to gel for several days. This is similar to previous reports of silk molecules of this size[22]. Furthermore, we found that the construct rapidly begins to precipitate out of solution during concentration, limiting the solution concentration we can achieve. As such, we focused our process development efforts on longer constructs and constructs containing CTD—the latter of which we anticipated would improve solution stability of the protein.

We produced hundreds of milligrams of 24B (~60 kDa). We were able to sufficiently concentrate 24B in 6M GdnSCN to make a fiber forming spin dope, and began small-scale spinning tests (Figure 6). Without high concentrations of chaotropes, 24B was found to be too unstable in aqeous solution to create a spin dope. In addition, the fibers produced from a chaotrope containing dope were too brittle to mechanically test. This led to our further investigation of the effect of terminal domains on the protein, as well as non-aqueous spin solvents (see below.)

Figure 6. Small scale fiber spinning test through extrusion of dissolved protein into a methanol coagulation bath. A syringe needle (top) is flowing silk dope into the coagulation and the solution is precipitating into a white fiber. This enables the testing the property potential of different constructs at a rapid pace.

Year 3

Summary

- Discovered promoter-dependent effects on silk secretion.
- Improved DNA assembly method for high throughput cloning and strain generation.
- Developed a strain generation system to secrete all known silk proteins.
- Improved strain productivity through multicopy insertion.
- Optimized process conditions for large scale silk protein production.
- Began large scale fiber spinning to optimize continuous production of yarns.

Modification of the Base Strain for Protein Secretion

Promoters. Several different promoters are used for the expression of heterologous proteins in *Pichia pastoris*. The AOX1 promoter is the most widely used promoter because of its strong protein expression under methanol induction. Whereas our initial work utilized pAOX1, we have discovered that *Pichia* promoters each have a unique impact on the ability of the cell to secrete a silk while also maintaining long-term cell metabolic health. In particular, higher molecular weight constructs secrete with very different efficiencies depending on the promoter used. Therefore, when troubleshooting silk secretion from *Pichia*, the promoter system used must be taken into account.

Secretion tags. Native silk NTDs contain a strongly predicted secretion signal that is often removed from the construct before heterologous expression[23]. We sought to understand the relative secretion efficiencies of the native silk secretion signal versus the commonly used alpha-mating factor (αMat) from *Saccharomyces cerevisiae*. We observed that a combination of αMat and the native secretion signal led to little or no protein secretion and that αMat was more effective at secreting silk in *Pichia* (Figure 7).

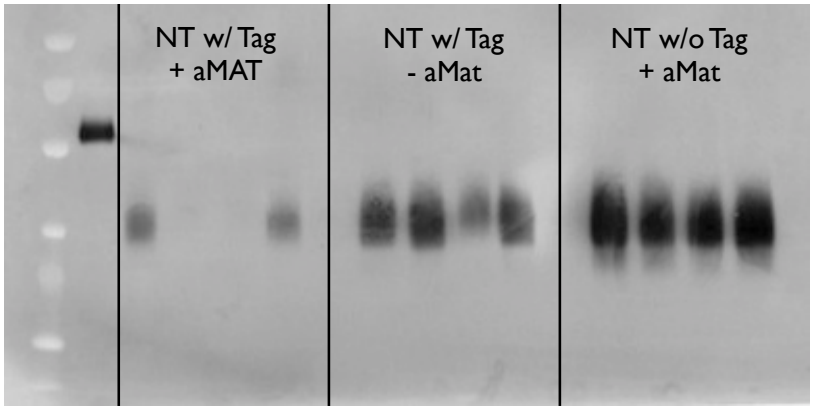

Figure 7. Western blot of supernatant for secreted (left) NTD with native secretion tag and αMat, (middle) NTD with native secretion tag and without αMat, and (right) NTD without native secretion tag and with αMat.

However, the challenge faced when using αMat is that it contains a pre-pro-peptide sequence. Miscleavage of the pre- and pro- forms can lead to additional residues preceding the protein of interest that could be subject to post-translational modifications such as glycosylation. We found that miscleaved, glycosylated silk constructs lead to brown colored fibers that exhibited unexpected mechanical properties (Figure 8). Constructs must be addressed individually to determine the proper cleavage site to avoid glycosylation.

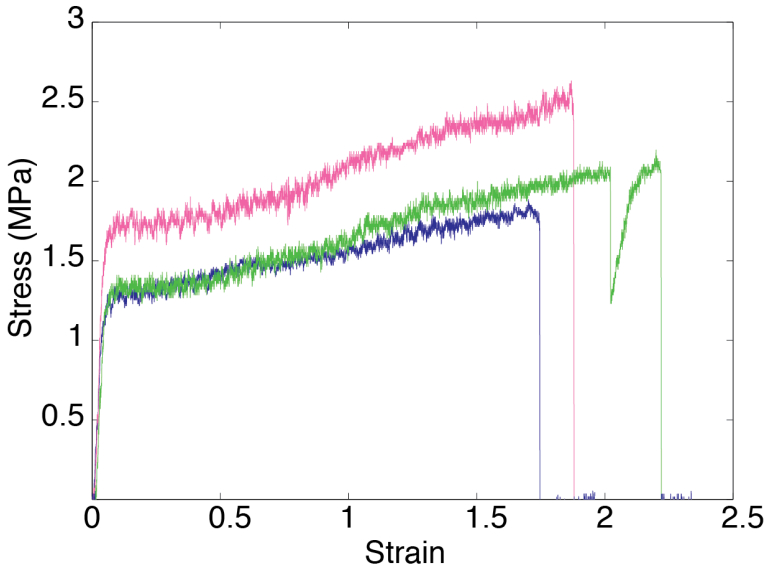

Figure 8. Stress-strain curve of 24B, glycosylated silk. Notice the extremely high strain to failure. The fibers were more gelatinous than non-glycosylated products.

High-throughput Strain Generation

Our initial DNA assembly technique was suitable for generating different molecular weight constructs of the same silk type within a several month timescale. We re-engineered our DNA assembly methodology for use with our robotics and automation setup. We developed a platform utilizing a modified version of the pseudo-scarless 2 antibiotic (2ab) method[24,25] to build repetitive DNA. We moved our *Pichia* transformation, growth, and screening to our robotics system. Now, when utilizing any silk DNA already available in house, we can produce a *Pichia* strain secreting a novel silk in just weeks. Alternatively, if new DNA synthesis is needed, the strain can be made in one to two months. Leveraging this platform, we have generated strains that secrete every known spider silk available in GenBank (>400 sequences.) We are using this platform to develop silks with mechanical properties of significant interest, as well as for the generation of novel silks with properties not available in nature. We are currently working on adding subsequent high-throughput purification and materials processing steps into this platform.

Multicopy Insertion

The most common methodology for improving the productivity of heterologous hosts is the insertion of multiple copies of the recombinant DNA of interest into the host genome[26]. This is often achieved by utilizing a strong antibiotic resistance marker and screening thousands to tens of thousands of transformants for multiple copy integration. Utilizing our high throughput screening system, we were able to demonstrate that multicopy integration is a viable means of improving silk secretion from *Pichia* (Figure 9). Because of the

altered metabolic load on the cells with multiple copies of the protein, process development in the fermentation process must be reinvestigated for each new strain.

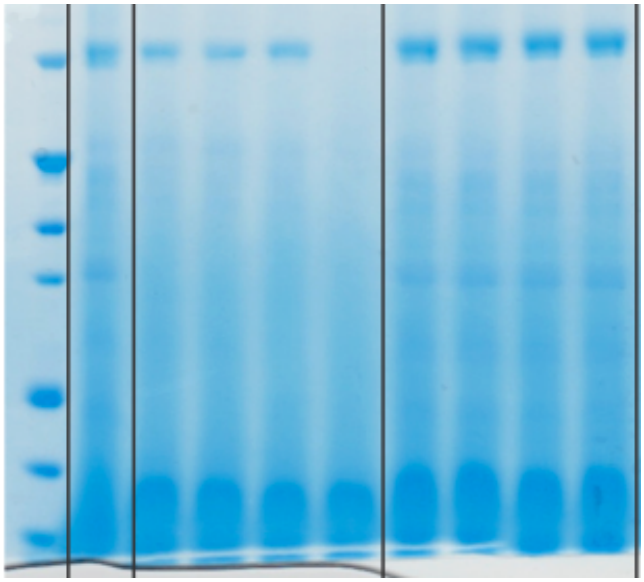

Figure 9. Coomassie stained gel demonstrating productivity improvement using multicopy transformation. Lanes 3-5 are supernatants from strains with lower copy numbers and lanes 7-10 are supernatants from strains with a higher copy number.

Large Scale Protein Production

Fermentation. Due to the geometric differences at increasing scales and the lack of feedback and control for pH, dissolved oxygen, and other variables, moving a silk-producing strain from the high-throughput plate screen to large-scale fermentation is a multi-step process. We are equipped with 1L, 2L, 5L, and 30L fermentation vessels in house, and currently develop and validate processes on the smaller volume vessels. Upon process validation, strains are moved to the larger vessels for process optimization and subsequently material production. We are currently achieving *O*(10) mg/L/hr productivities of silk protein through process optimizations alone.

Purification. Generating such large volumes of supernatant requires purification strategies that are amenable to that scale. Furthermore, the economics of these purification strategies must be considered for future scale-up (*e.g.* column chromatography is prohibitively expensive at large scale.) In an attempt to purify small amounts of material for property potential testing, we explored affinity chromatography of 6xHIS tagged silk proteins. We were faced with the same difficulties as have been previously reported[27,28], in that silk proteins tend toward structure formation that masks the affinity tag and makes column binding difficult. Despite extended investigation, it is unclear to us why

constructs expressed intracellularly were more amenable to affinity chromatography (see Year 1). We suspect this is a result of the protein aggregation behavior seen in the fermenter upon secretion (see Year 2).

Currently for purification, we discovered that a combination of precipitation and filtration that we can utilize at scale (Figure 10). We have found purification schemes to be highly dependent on the silk construct being explored, as well as dependent on the structured state of the construct. Furthermore, we have found that when scaling up purification strategies to larger equipment that necessitate liquid pumping, the shear effects within the process can induce aggregation of the silk protein and inhibit purification. This is not uncommon for recombinant proteins, but usually accounts for a small loss in product[29]. With silk constructs, we have found it to be completely detrimental to a processing operation.

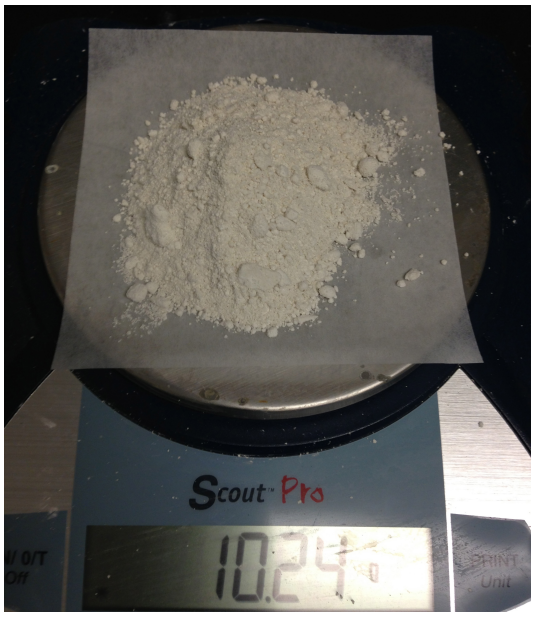

Figure 10. ~10g of silk protein produced from a large fermentation vessel and purified with scalable equipment.

Fiber Spinning

Spin dope preparation. Both spider silk and silkworm silk are naturally spun from aqueous conditions. For reasons of safety and economics at scale, it would be beneficial to spin recombinant silk fibers from aqueous conditions as well. We have found that our constructs, both with and without terminal domains, exhibit a concentration-dependent aggregation in an aqueous environment. This behavior makes it extremely difficult to concentrate the protein solutions to levels from which fibers can be spun (~20% w/w). Although data in the literature suggests that terminal domains should help with this concentration dependent stability, we suspect that proper folding of the terminal domains is necessary for this behavior. It is currently unknown if the terminal domains of our constructs are

properly folded after secretion and processing. Therefore, we switched to non-aqueous and scalable, solvents for spin dope preparation. Future work will entail determining if native quality spider silk fibers can be spun from non-aqueous solvents.

Fiber spinning. We have identified a silk construct that demonstrates sufficient property potential of interest to us to scale protein production to allow repeated testing on the spinline (Figure 11, left). Whereas this protein is only a fraction of the molecular weight of a full-length spider silk protein, it serves a platform to perform process development on a full-scale fiber spinline. We have found that the conditions for syringe pump extrusion of silk fibers often seen in literature do not translate to industrial scale wet spinning. The magnitudes of the forces and time scales and the different geometric constraints for spinning require different pumping speeds, reeling speeds, coagulant strengths, and filament counts than would be utilized with a syringe pump. In particular, flow instabilities are more prevalent when using small aspect ratio spinnerets instead of long syringe needles. We are currently creating spools of fiber on a spinline, and are base-lining the process for maximum mechanical property potential (Figure 11, right). Future work will involve working through this process with silk constructs on the molecular size scale of native silk protein.

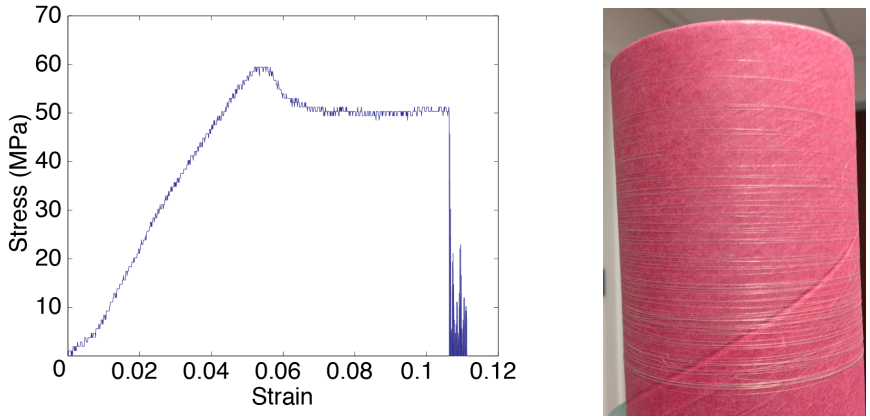

Figure 11. (left) Stress-strain curve of undrawn, property potential fibers of recombinant silk. (right) Spool of drawn fibers spun on a commercial spinline.

Personnel

David Breslauer, PhD
Daniel Widmaier, PhD
Ethan Mirsky, PhD
Joshua Kittleson, PhD

Patent Disclosures

U.S. Patent Application No. 61/716,890, “Cellular Reprogramming for Product

Optimization." Daniel Widmaier and David Breslauer.

Under filing, "Methods and Compositions for Synthesizing Improved Silk Fibers." Daniel Widmaier, David Breslauer, and Joshua Kittleson.

Interactions

Refactored Materials is committed to engaging with the academic and industrial community. Our scientific advisory board includes Prof. David Kaplan (Tufts), Prof. Sam Hudson (NCSU), Prof. Chris Voigt (MIT), Prof. Travis Bayer (Oxford), and Prof. Susan Muller (UC Berkeley), all of whom we interact with regularly. We are industrial members of SynBERC, the NSF Synthetic Biology Engineering Research Center, as well as the Synthetic Fibers and Yarns Association. We regularly attend and participate in the Silk workshops when they are held.

Bibliography

(1) Prince, J. T.; McGrath, K. P.; DiGirolamo, C. M.; Kaplan, D. L. *Biochemistry* **1995**, *34*, 10879.

(2) Randolph V. Lewis, M. H., Srinivas Kothakota, and Maurille J. Fournier *Protein Expressing and Purification* **1996**, *7*, 6.

(3) Fahnestock, S. R.; Bedzyk, L. A. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 33.

(4) Fahnestock, S. R.; Irwin, S. L. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 23.

(5) Arcidiacono, S.; Mello, C.; Kaplan, D.; Cheley, S.; Bayley, H. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 31.

(6) Fukushima, Y. *Biopolymers* **1998**, *45*, 269.

(7) Huemmerich, D.; Helsen, C. W.; Quedzuweit, S.; Oschmann, J.; Rudolph, R.; Scheibel, T. *Biochemistry* **2004**, *43*, 13604.

(8) Ayoub, N. A.; Garb, J. E.; Tinghitella, R. M.; Collin, M. A.; Hayashi, C. Y. *PLoS One* **2007**, *2*, e514.

(9) Ellis, T.; Adie, T.; Baldwin, G. S. *Integr Biol (Camb)* **2011**, *3*, 109.

(10) Scheller, J.; Guhrs, K. H.; Grosse, F.; Conrad, U. *Nat Biotechnol* **2001**, *19*, 573.

(11) Lazaris, A.; Arcidiacono, S.; Huang, Y.; Zhou, J. F.; Duguay, F.; Chretien, N.; Welsh, E. A.; Soares, J. W.; Karatzas, C. N. *Science* **2002**, *295*, 472.

(12) Huemmerich, D.; Scheibel, T.; Vollrath, F.; Cohen, S.; Gat, U.; Ittah, S. *Curr Biol* **2004**, *14*, 2070.

(13) Cregg, J. M.; Barringer, K. J.; Hessler, A. Y.; Madden, K. R. *Mol Cell Biol* **1985**, *5*, 3376.

(14) Cregg, J. M.; Madden, K. R.; Barringer, K. J.; Thill, G. P.; Stillman, C. A. *Mol Cell Biol* **1989**, *9*, 1316.

(15) Cregg, J. M.; Cereghino, J. L.; Shi, J.; Higgins, D. R. *Molecular biotechnology* **2000**, *16*, 23.
(16) Heiss, S.; Maurer, M.; Hahn, R.; Mattanovich, D.; Gasser, B. *Applied microbiology and biotechnology* **2012**.
(17) Love, K. R.; Politano, T. J.; Panagiotou, V.; Jiang, B.; Stadheim, T. A.; Love, J. C. *PloS one* **2012**, *7*, e37915.
(18) Love, K. R.; Panagiotou, V.; Jiang, B.; Stadheim, T. A.; Love, J. C. *Biotechnol Bioeng* **2010**, *106*, 319.
(19) Eisoldt, L.; Thamm, C.; Scheibel, T. *Biopolymers* **2012**, *97*, 355.
(20) Hagn, F.; Thamm, C.; Scheibel, T.; Kessler, H. *Angew Chem Int Ed Engl* **2011**, *50*, 310.
(21) Xia, X. X.; Qian, Z. G.; Ki, C. S.; Park, Y. H.; Kaplan, D. L.; Lee, S. Y. *Proc Natl Acad Sci U S A* **2010**, *107*, 14059.
(22) Arcidiacono, S.; Mello, C. M.; Butler, M.; Welsh, E.; Soares, J. W.; Allen, A.; Ziegler, D.; Laue, T.; Chase, S. *Macromolecules* **2002**, *35*, 1262.
(23) Hagn, F.; Eisoldt, L.; Hardy, J. G.; Vendrely, C.; Coles, M.; Scheibel, T.; Kessler, H. *Nature* **2010**, *465*, 239.
(24) Leguia, M.; Brophy, J. A.; Densmore, D.; Asante, A.; Anderson, J. C. *J Biol Eng* **2013**, *7*, 2.
(25) Kodumal, S. J.; Patel, K. G.; Reid, R.; Menzella, H. G.; Welch, M.; Santi, D. V. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 15573.
(26) Marx, H.; Mecklenbrauker, A.; Gasser, B.; Sauer, M.; Mattanovich, D. *FEMS Yeast Res* **2009**, *9*, 1260.
(27) Mello, C. M.; Soares, J. W.; Arcidiacono, S.; Butler, M. M. *Biomacromolecules* **2004**, *5*, 1849.
(28) Gaines, W. A.; Marcotte, W. R., Jr. *AATCC Rev* **2011**, *11*, 75.
(29) Maa, Y. F.; Hsu, C. C. *Biotechnol. Bioeng.* **1997**, *54*, 503.